Appendix A

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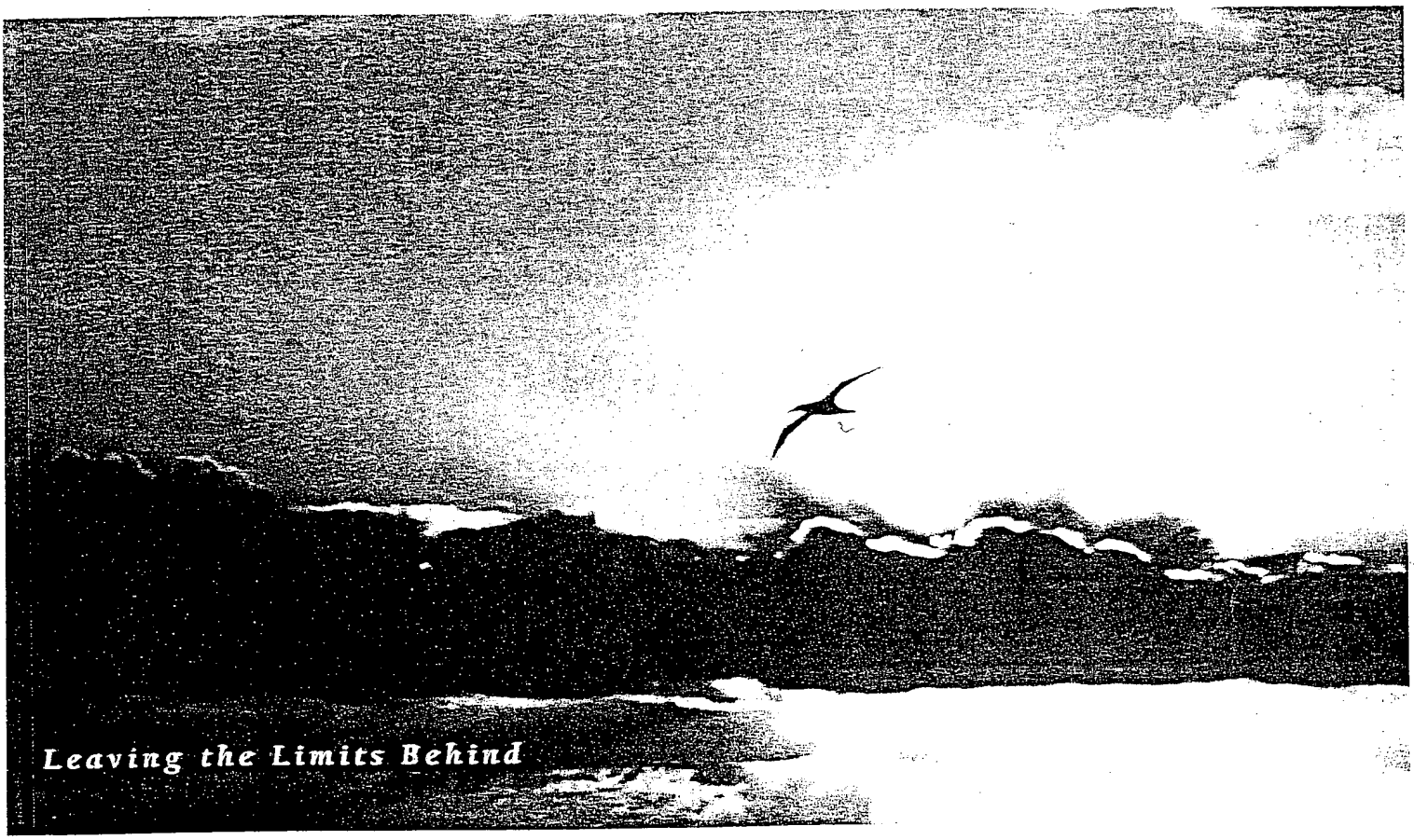
Biochemicals

1994 Catalog



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Primary Antibodies

Antibody	Specificity and Notes	Species Specificity
Antibodies for Neuroscience and Signal Transduction Research (continued)		
Anti-Alzheimer precursor protein A4, formalin grade (Pre-A4) (clone 22C11) Cat. No. 1285 262	The antibody was produced by immunization with the fusion protein pre-$A4_{695}$, and reacts with the human Alzheimer precursor protein A4. It shows cross-reactivity with the pre-A4 molecules from fish, rat, mouse, and monkey. **Stability:** Lyophilizate is stable at +4°C. Reconstituted antibody solution (200 µg/ml in water) is stable at –20°C. Avoid repeated freezing and thawing.	human, mouse, r fish, monkey
Anti-β-Amyloid, Alzheimer Cat. No. 1381 431	The antibody reacts with plaques in brain cryosections of Alzheimer patients. **Stability:** Lyophilizate is stable at +4°C. Reconstituted antibody solution (200 µg/ml in water) is stable at –20°C. Avoid repeated freezing and thawing.	human
NEW **Anti-Ca^{2+}/calmodulin-dependent protein kinase type II** (clone 6G9) Cat. No. 1481 703	Recognizes the α-subunit of Ca^{2+}/calmodulin-dependent protein kinase II and reacts with tissue from all mammalian species tested, as well as with chicken and frog tissues. **Stability:** Stable at –20°C.	mammals, chicke frog
Anti-calcitonin gene-related peptide, human Cat. No. 1295 241	To obtain the polyclonal antiserum, rabbits were immunized with calcitonin gene-related peptide. The antibody is suitable for detection of CGRP on brain sections. **Stability:** Lyophilizate is stable at +4°C. Reconstituted antibody solution (200 µg/ml in water) is stable at –20°C. Avoid repeated freezing and thawing.	human, mouse, r chicken
Anti-L-CAM/Uvomorulin (clone 6F9) Cat. No. 1441 892	The antibody specifically recognizes the 120 kD and the 80 kD band of L-CAM/Uvomorulin (Arc-1 E-cadherin cell-CAM 120/80) in man and rabbit. L-CAM/Uvomorulin staining is confined to the lateral border of epithelial cells and, within the intestine, shows more intense concentrations in the area of the junctional complex. As a positive control, the cell line MCF-7 can be used. **Stability:** Lyophilizate is stable at +4°C. Reconstituted antibody solution (200 µg/ml in water) is stable at –20°C. Avoid repeated freezing and thawing.	human, rabbit
Anti-choline acetyltransferase (ChAT) (clone 11-255) Cat. No. 770 981	The antibody reacts with choline acetyltransferase from monkey, pig, rat, and mouse. **Stability:** Lyophilizate is stable at –20°C. Store reconstituted antibody solution at +4°C. **Do not freeze.**	mouse, rat, pig, monkey
Anti-choline acetyltransferase, human (ChAT) (clone 1.B3.9B3) Cat. No. 1464 272	The antibody reacts with choline acetyltransferase from man, rat, and pig. The antibody can be used for investigating the decrease of the cholinergic system in Alzheimer's disease. **Stability:** Stable at +4°C.	human, rat, pig
Anti-chromogranin A (clone LK2H10) Cat. No. 1199 021	The antibody recognizes the 68 kD protein, chromogranin A, found exclusively in secretory storage granules of almost all neuroendocrine cells. The antibody binds small cell carcinoma of the lung, Merkel cell carcinomas and neuroblastomas weakly; binds most other endocrine-derived tumors strongly. Primary antibody for immunohistochemical detection and characterization of normal endocrine cells and endocrine-derived tumor cells [Lloyd, R.V. and Wilson, B.S. (1983) *Science* 222, 628-630; Wilson, B.S. and Lloyd, R.V. (1984) *Am. J. Pathol.* 115, 458-468]. **Stability:** Stable at +4°C for prolonged storage, aliquot and store at –20°C or colder. Avoid repeated freezing and thawing.	human, monkey, pig
Anti-CNP (2',3'-cyclic nucleotide 3'-phosphodiesterase) (clone 11-5B) Cat. No. 1442 007	The antibody reacts with both CNPase 1 and CNPase 2 (2',3'-cyclic nucleotide 3'-phospho-diesterase) and is used as an oligodendrocyte and Schwann cell marker. **Stability:** Lyophilizate is stable at +4°C. Reconstituted antibody solution (200 µg/ml in water) is stable at –20°C. Avoid repeated freezing and thawing.	human, mouse, ra rabbit, bovine, dog sheep
NEW **Anti-dystrophin** (clone 1808C) Cat. No. 1558 722	The antibody recognizes dystrophin, the 427 kD product of a 14 kb transcript encoded by the Duchenne muscular dystrophy gene locus on chromosome Xp21. It does not label human Duchenne muscle or mouse mdx (murine muscular dystrophy) tissue, and it does not cross-react with proteins closely related to dystrophin (*e.g.*, C-protein, α-actin, or human muscle spectrin). **Reference:** Hoffman, E.P., Brown, R.H. and Kunkel, L.M. (1987) *Cell* 51,919. **Stability:** The antibody is stable for 18 months at +4°C or 2 years at –20°C. Once reconstituted, store the antibody in aliquots at –20°C to avoid repeated freezing and thawing. Avoid gross bacterial contamination.	human, rat, mouse other mammalian, chicken, frog
Anti-β-Endorphin (clone 3-E7) Cat. No. 1089 170	The antibody reacts with the NH2-terminal Tyr-Gly-Gly-Phe-sequence of human β-endorphin. Therefore, there is a high cross-reactivity with homologues with the same sequence like (Met)-enkephalin and (Leu)-enkenhalin and many opioid peptides. The antibody reacts with β-endorphin from pig and camel. **Stability:** Lyophilizate is stable at –20°C. Store the reconstituted antibody solution at +4°C.	human, pig, camel
Anti-$GABA_A$ receptor, α-chain (Anti-γ-aminobutyric acid) (clone bd 24) Cat. No. 1381 440	The antibody reacts with the α-chain of $GABA_A$ receptor from cow and rat. **Stability:** Lyophilizate is stable at +4°C. Reconstituted antibody solution (200 µg/ml in water) is stable at –20°C. Avoid repeated freezing and thawing.	human, bovine
Anti-$GABA_A$ receptor, β-chain (Anti-γ-aminobutyric acid) (clone bd 17) Cat. No. 1381 458	The antibody reacts with the β-chain of $GABA_A$ receptor from cow, man, and rat. **Stability:** Lyophilizate is stable at +4°C. Reconstituted antibody solution (200 µg/ml in water) is stable at –20°C. Avoid repeated freezing and thawing.	human, rat, bovine cat

B = Southern/northern/dot blots
C = Cryosections
CD = Cluster of Differentiation
E = ELISA
FC = Flow cytometry
IC = Immunocytochemistry
IHC = Immunohistochemistry
IP = Immunoprecipitation
N = Neutralizing
P = Paraffin sections
S = Histological sections
W = Western (proteins) blots

Note: Listed applications are tested by Boehringer Mannheim. Any application not listed does not necessarily indicate that the antibody does not work in that application.

Ig Class	Application	Working Conc. (µg/ml)	No. of Tests	Form	Cat. No.	Pack Size	Price ($)
Mouse IgG_1 (monoclonal)	C, P, W	5–10	250–1,000 (S)	Lyophilized	1285 262 1272 829	50 µg ☐	252.00
Rabbit Ig (polyclonal)	C, P, W	10–20 (S), 20 (W)	50–500 (S)	Lyophilized	1381 431 1384 082	100 µg ☐	244.00
Mouse IgG_1 (monoclonal)	C, W	10 (IC, W)	200 (IC)	Lyophilized	1481 703 1484 711	200 µg ☐	237.00
Rabbit IgG (polyclonal)	C, P	5–10	250–500 (S)	Lyophilized	1295 241 1284 576	50 µg ☐	202.00
Mouse IgG_1 (monoclonal)	C, W, IC, E	1–4 (IC)	250–500 (IC)	Lyophilized	1441 892	50 µg	Inquire
Rat IgG (monoclonal)	C, P (limited application)	2.5–4 (S)	125–200 (S) 500–800 (S)	Lyophilized Lyophilized	770 981 770 990 755 702	10 µg 40 µg ☐	116.00 329.00
Mouse IgG_1 (monoclonal)	C, W P (limited application)	10–20 (CS)	100–200 (S)	Lyophilized Lyophilized	1464 272 1372 432 1372 025	50 µg 100 µg ☐	180.00 438.00
Mouse IgG_1 (monoclonal)	C, P	1–10 (S)	2,500–25,000 (S)	Solution, 500 µl	1199 021 1199 030	500 µg ☐	330.00
Mouse IgG_1 (monoclonal)	C, W, IC	10 (IC)	100–200 (IC)	Lyophilized	1442 007 1442 015	100 µg	195.00
Mouse IgG_1 (monoclonal)	IHC				1558 722 1558 749	100 µg ☐	155.00
Mouse IgG_{2a} (monoclonal)	E	1 (E)	400 (E)	Lyophilized	1089 170 1091 395	40 µg ☐	216.00
Mouse IgG_1 (monoclonal)	C, W, IP P (limited application)	10–20 (S), 20 (W)	100–200 (S)	Lyophilized	1381 440 1381 261	100 µg ☐	249.00
Mouse IgG_1 (monoclonal)	C, W, IP P (limited application)	10–20 (C), 20 (W)	50–500 (C)	Lyophilized	1381 458 1381 288	100 µg ☐	249.00

☐ Indicates special bulk quantity availability, see page ii for details.

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Appendix B

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REPRODUCTIVE IMMUNOLOGY

Journal of Reproductive Immunology 25 (1993) 89–102

Inhibition of fertility in female mice by immunization with a B-cell epitope, the synthetic sperm peptide, P10G

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(Accepted 18 August 1993)

Abstract

Human patient sera containing antisperm antibodies (from vasectomized men and infertile women) immunologically react with the synthetic peptide P10G (PGGGTLPPSG), and affinity purified antibodies to P10G (anti-P10G) react with human spermatozoa (O'Rand et al., 1990). In this study P10G was used to elicit antibodies and the effect of the antibodies on fertility in female mice determined. The P10G sequence is derived from the 14-kDa rabbit sperm autoantigen, RSA (O'Rand and Widgren, 1990). The results of this study demonstrate that female mice can become infertile when immunized with the synthetic peptide P10G conjugated to the carrier protein keyhole limpet hemocyanin (KLH). However, the results also show that it is important to distinguish those mice with high serum antibody levels from those with lower levels. Infertility was clearly apparent in the high titer subgroup with an 80% decrease in pregnancy rate during the last of three matings and a 71% decrease in litter size over all three matings when compared to the low titer subgroup or the control groups. Significantly, mice immunized with P10G without carrier protein show no detectable antigen specific proliferation of lymph node cells in response to 100 μM of the peptide. P10G is not a T-cell epitope, but rather a B-cell epitope and it does not elicit an autoimmune response in the female mouse. This demonstration in mice is an important first step in the development of a safe human immunocontraceptive vaccine.

Key words: Sperm antigen; Contraceptive vaccine; Sperm peptide; B-cell epitope

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1. Introduction

Induction of infertility in experimental animals by immunization with spermatozoa or sperm specific antigens has been demonstrated numerous times in previous studies but always with varying degrees of success. Mice have received considerable attention because of the variety of inbred strains available, but rats, rabbits and guinea-pigs have also been utilized (mice: Tung, et al., 1979; Anderson and Madrigal, 1985; rats: Tsunoda and Chang, 1976; Vanage et al., 1992; rabbits: Menge, 1968; Goldberg, 1973; O'Rand, 1980; O'Rand and Fisher, 1988; guinea-pigs: Katsh, 1959; Primakoff et al., 1988). Ever since the earliest study of Baskin (1932), it has also become apparent that infertility in some women may have an antisperm antibody basis (Isojima et al., 1988; Anderson and Madrigal, 1985). The most successful experimental study to date has been that of Primakoff et al., (1988) in which complete infertility resulted when high titer antibodies to the guinea-pig sperm antigen PH-20 were produced in female guinea-pigs. The infertility was reversible and the authors noted that as serum titers declined the probability of an individual female becoming pregnant increased.

The search for agents which can induce infertility in humans by vaccination without unwanted side-effects is complicated by the need to balance immunological potency with gamete specificity and delivery. Ideally, human sperm-specific proteins or peptides could be found which would elicit immune responses and deliver antibodies to the reproductive tract in order to block sperm function. However, the necessity of testing in animal models prior to human usage dictates the utilization of animal proteins or peptides with similar structural and functional properties. To this end, the present study has utilized a synthetic decamer peptide, P10G (PGGGTLPPSG), to which patient sera containing antisperm antibodies (from vasectomized men and infertile women) immunologically react in ELISA and whose affinity-purified antibodies (anti-P10G) react with human spermatozoa in ELISA (O'Rand et al., 1990). The P10G sequence is derived from the 14-kDa rabbit sperm autoantigen, RSA (O'Rand and Widgren, 1990). RSA is a family of four rabbit sperm autoantigens which function as lectin-like zona binding proteins on the surface of spermatozoa whose antiserum will inhibit fertilization both in vivo and in vitro (O'Rand et al., 1988). Autoantiserum to rabbit spermatozoa (male rabbits immunized with their own spermatozoa) reacts with P10G in ELISA and anti-P10G antibodies react with the parent 14-kDa RSA molecule in ELISA (O'Rand and Widgren, 1990). An analog of RSA is also present on mouse spermatozoa and mouse anti-rabbit RSA antiserum will inhibit mouse sperm-zona binding in vitro (Richardson et al., 1991). Consequently, based on the autoimmunogenicity of P10G in humans and the presence of an analog of RSA in mice, P10G would appear to be a good candidate for testing in mice as a B-cell epitope in a potential contraceptive-vaccine.

2. Materials and methods

2.1. Animals

In this study BALB/cBy female mice and CD-1 male mice were purchased from Charles River Co., MA. The virgin females were 6 weeks old at the beginning of the study and tagged for individual identification throughout the study. The females were housed 3 per cage. The males were proven breeders.

For mating experiments, 1 male was housed with 3 females for 24 h. Females with vaginal plugs were removed to separate cages and the unplugged females moved to a new male. This schedule was continued for 8 days. The same males were used for both immune and control groups. Between 18–23 days after the plug was observed, the mice were checked once or twice daily for pups. Three matings were carried out during this study such that by the end of the experiment the females were 38 weeks old.

2.2. Antigen preparation

In this study the peptide P10G (PGGGTLPPSG; U.S. patent No. 5,175,148) was synthesized by Peninsula Labs (Belmont, CA) and used as the B-cell epitope in the test immunogen. It was conjugated to the carrier protein KLH (keyhole limpet hemocyanin) by coupling to amide groups with EDC (1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride as indicated by the manufacturer (Pierce Chemical Co., Rockford, IL). Following conjugation the P10G-KLH was purified by gel filtration in PBS (83 mM phosphate buffer + 0.9 M NaCl; pH 7.0).

2.3. Immunization schedule

The immunization schedule is indicated in Table 1. In experiment 1, only the first 4 injections were given. Each experimental mouse received 50 μg of P10G-KLH in 200 μl and each KLH control mouse received 50 μg of KLH only in 200 μl. The first injection consisted of 100 μl antigen and 100 μl Freund's complete adjuvant and each subsequent injection contained 100 μl of antigen and 100 μl of incomplete adjuvant, except for the last injection which consisted of 50 μg of P10G (non-conjugated) in 100 μl which was given i.p. into each experimental mouse.

2.4. Enzyme linked immunosorbent assay (ELISA) for serum antibody

To determine the presence of antibodies to P10G in the immunized mice, P10G was conjugated to thyroglobulin by the same procedure described for

KLH and 0.25 μg of the conjugate plated/well of an ELISA plate. Control wells contained 0.25 μg of thyroglobulin only. From each serum collection (Table 1), two dilutions, 1/100 and 1/800, were tested for each immune and control mouse. The assay was developed with peroxidase-labeled goat anti-mouse IgG, IgM, IgA at a 1/1000 dilution followed by TMB substrate (Kirkegaard & Perry, Gaithersburg, MD). Duplicate values were averaged and the thyroglobulin values subtracted from the P10G-thyroglobulin values for each serum at each dilution tested. Four wells (2 with P10G-thyroglobulin and 2 with thyroglobulin) treated with secondary antibody only were averaged and served as background controls for each plate. These values were subtracted from all the wells on the plate.

To determine the presence of antisperm antibodies in the sera of immunized mice, mouse epididymal spermatozoa were fixed to ELISA plates. ELISA plates were first primed overnight in 0.1% glutaraldehyde in water at 4°C, rinsed, and 10^5 epididymal mouse spermatozoa in 0.1 ml placed in each well. The plates were centrifuged at 1000 rev./min for 5 min and 100 μl of 0.1% glutaraldehyde added, incubated for 30 min at room temperature, rinsed and blocked with 1% bovine serum albumin. The assay was developed as described for P10G. Values from control wells with pooled sera from KLH only immunized mice were subtracted from test wells.

2.5. Experimental design

The first experiment (Exp. 1; Table 3) was a pilot experiment and consisted of three P10G-KLH immunized mice and three KLH only immunized control mice. The mice received the first 4 immunizations (I) indicated in Table 1 and were mated only once (M1). Since 67% (2/3) of the control mice and 0% (0/3) of the experimental mice became pregnant, a larger test group seemed appropriate. Therefore experiment 1 was terminated and experiment 2 started. Experiment 2 consisted of 12 mice immunized with P10G-KLH, 12 mice immunized with KLH only, and 12 mice which were not immunized. After the last immunization the experimental group received two additional matings (M2, M3, Table 1) and a final serum collection (B4). The KLH only and no immunization groups were not used for the M2 and M3 matings as the low titer P10G group served as a control for the high titer group (see Results section). The Student's *t*-test was used for statistical analysis of the data

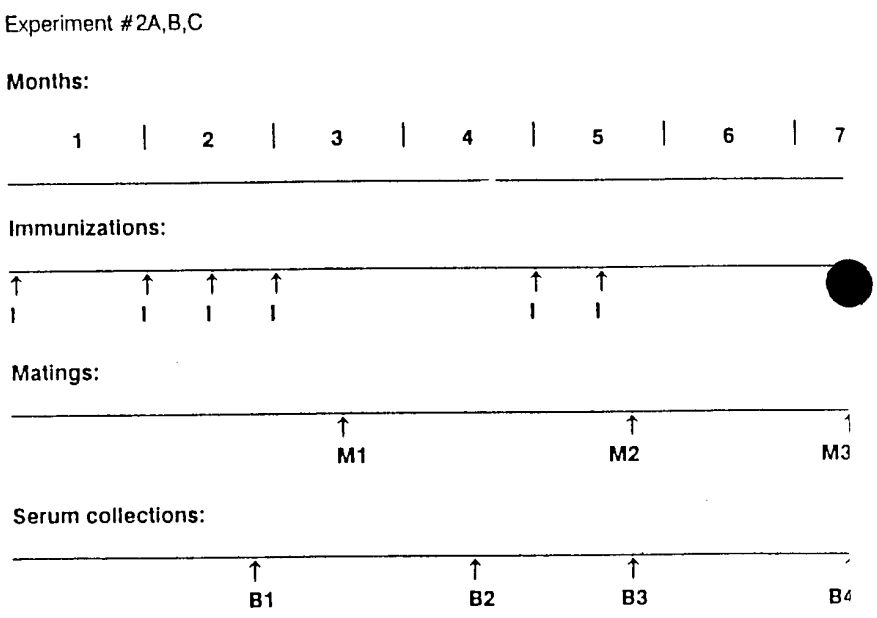
2.6. In vitro sperm-zona binding

Anti-P10G-KLH sera obtained from immunized mice were tested for their effect on mouse sperm-zona binding using the binding assay described by Bleil and Wassarman (1990) except that 2% BSA and BWW were used as described by Richardson et al. (1991). Immune and control antisera were diluted 1/25 with BWW before use. For each serum 10–20 cumulus-free oocytes were tested in 50 μl medium containing 2 μl serum and 12 500 spermatozoa. The number of spermatozoa bound per oocyte was recorded and is reported as a percentage of PBS control. Data from separate experiments were not combined.

2.7. T-cell reactivity

To test for T-cell reactivity to P10G (T-cell epitope), five female BALB/cBy mice were each immunized with 1 mM P10G (non-conjugated) mixed 1:1 with complete Freund's adjuvant in 100 μl. Eighteen days after the injection serum was collected and the mice were sacrificed for ovarian histology and antigen-specific lymphocyte proliferation against 1.0, 10.0, and 100.0 μM of P10G as described (Rhim et al., 1992). As a positive control in the lymphocyte proliferation assay, the lymph node cells were also tested against purified protein derivatives of *Mycobacterium* in complete Freund's adjuvant.

Table 1
Experimental time course of immunizations, matings and serum collections (Experiments 2A,B,C)



3. Results

Table 1 describes the immunizations (I), matings (M) and serum collections (B) conducted during the current study. In the first experiment (Exp. 1; Table 3) three P10G-KLH immunized mice and three KLH only immunized control mice were used. The mice received 4 immunizations (the first 4 indicated in Table 1) and then were mated (M1). Sixty-seven percent (2/3) of the control mice and 0% (0/3) of the experimental mice became pregnant (Table 3). Experiment 1 was terminated and experiment 2 started. Experiment 2 contained 12 mice immunized with P10G-KLH, 12 mice immunized with KLH only, and 12 mice which were not immunized. The 6 immunizations (I) are shown in Table 1, the first 4 following the same schedule as in experiment 1. After the first 3 immunizations, the first serum collection (B1, Table 1) showed that all 12 mice responded to the P10G-KLH immunogen, but with serum O.D. values which varied from 0.04–2.74 at a 1/800-dilution of the serum (Table 2).

Following the fourth immunization, the mice were mated (M1; Table 1). The results indicate that there were no significant differences between the experimental group and KLH or no injection control groups (Exp. 2A, Table 3; 8 pregnant (4.7 pups/litter) vs. 9 pregnant (4.2 pups/litter) for KLH). The second serum collection (B2) again revealed a wide range in O.D. values indicating various degrees of responsiveness to P10G. Two additional immunizations were given, the last consisting of 50 μg of the unconjugated P10G peptide (see Materials and methods). Analysis of the ELISA data from the third serum collection (B3) 10 days after the last immunization revealed increased titers to P10G in the majority of the mice (Table 2). Additionally, it was observed that 6 of the mice had O.D. values at 450 nm of a 1/800-dilution of the serum above 0.9, and 6 of the mice had values below 0.9. These were designated as high and low titer subgroups, respectively. Without further immunizations, 2 additional matings were conducted to compare the high titer subgroup with the low titer subgroup and the group as a whole with the original KLH only control and no injection control groups.

Table 3 shows the results of the second mating (Exp. 2B) and third mating (Exp. 2C) of the 12 experimental mice. Although there was a slight drop in the number of pups/litter during experiment 2C, over the entire 39 mating cycles, there was no significant difference ($P = 0.33$) between the pups/litter in the P10G group (2.8 ± 2.2) and all control groups during 27 mating cycles (4.4 ± 1.6).

However, further analysis revealed that if the high titer and low titer subgroups (B4) were compared during the last mating (M3), then a significant difference between the subgroups was observed. Table 4 shows that there was a significant difference between the serum 1/800 O.D. value of the high titer subgroup and the low titer subgroup (0.23 ± 0.05 vs. 0.11 ± 0.05; $P < 0.002$). Moreover, there was a significant difference ($P < 0.004$) between the pups/litter (0.3 ± 0.8) in the high titer group and the pups/litter in the low titer group (4.0 ± 2.3). Significantly there was an 80% decrease

Table 2
O.D. value at 450 nm of a 1/800-dilution of each mouse serum collection

Mouse No.	Serum collection			
	B1	B2	B3	B4
M1	1.12	0.39	0.89	0.17
M2	1.20	0.14	0.23	0.07
M3	0.04	0.05	0.25	0.09
M4	2.74	1.43	2.31	0.25
M5	0.47	0.24	1.18	0.21
M6	1.33	1.00	2.04	0.32
M7	1.04	0.97	0.95	0.20
M8	0.67	1.02	0.51	0.15
M9	0.44	0.27	1.30	0.19
M10	0.52	0.35	0.49	0.03
M11	0.38	0.40	0.57	0.14
M12	1.04	1.59	1.93	0.21

Table 3
Pregnancy outcome

Experimental	No. mice	No. pregnant	No. pups	% Pregnant	Pups/litter
Exp. 1	3	0	0	0	0
Exp. 2A[a]	12	8	56	67	4.7
Exp. 2B	12	10	53	83	4.4
Exp. 2C	12	6	26	50	2.2
Total	39	24	135	62	2.8 ± 1.2*
Control					
Exp. 1 KLH	3	2	9	67	3.0
Exp. 2 KLH	12	9	50	75	4.2
None	12	8	73	67	6.1
Total	27	19	132	70	4.4 ± 1.6

[a]The same 12 mice were used in experiment 2A,B, and C.
*Not significantly different from control $P = 0.33$.

in the pregnancy rate (Table 4). The effect of high antibody titers to P10G was also apparent when the results from all three matings (M1,M2,M3) were analyzed. Table 5 shows the same high and low titer subgroups as Table 4, but additionally demonstrates that over the course of the entire experiment there were significantly fewer ($P < 0.003$) pups/litter (1.7 ± 1.7) in the high titer subgroup than in the low titer subgroup (5.8 ± 1.9), a 71% decrease. The low titer subgroup is not significantly different from any of the control groups shown in Table 3. The data in Table 5 is shown graphically in Fig. 1. The 6 mice with the highest serum 1/800 O.D. values have, on average, the fewest pups/litter. Clearly mouse M6 stands out as an exception.

Eight of the sera from the 12 experimental mice were tested for their effect on mouse sperm egg-binding in vitro. The results are shown in Fig. 2. These sera were compared to a pool of control sera from non-injected mice (C12; N) and a pool of control sera from mice injected with KLH only (C12; K). Mice from both the high titer subgroup (i.e., M9, M5, M4, M6) and low titer subgroup (M10, M2, M3, M11) all significantly inhibited sperm-zona binding (Fig. 2). Even the mouse with the lowest antibody titer (M10) was able to inhibit sperm-zona binding almost completely.

All of the sera from the last serum collection (B4) were tested in ELISA for reactivity to epididymal mouse spermatozoa. With the exception of mouse M9, all the sera were positive for the presence of antisperm antibodies. However, there was no significant difference in the ELISA serum 1/800 dilution value (O.D., 450 nm) between the high titer subgroup (0.227 ± 0.21) and the low titer subgroup (0.222 ± 0.17). Each immune serum was also tested in a Western blot assay (Nikolajcyk and O'Rand, 1992) for reactivity to mouse spermatozoa, but no significant differences between immune and control sera could be demonstrated.

The synthetic peptide P10G was also tested for its ability to elicit a T-cell response in mice and for its ability to cause any ovarian pathology. The

Table 4
Pregnancy outcome and serum antibody level for the last mating (Experiment 2C)

Mouse No.	Serum O.D.value 1/800	No. pups
High titer		
M6	0.32	0
M4	0.25	2
M5	0.21	0
M12	0.21	0
M7	0.20	0
M9	0.19	0
Average	0.23 ± 0.05 ($P < 0.002$)*	0.3 ± 0.8 ($P < 0.004$)*
No. pregnant		1
% pregnant		17 80%↓
Low titer		
M1	0.17	3
M8	0.15	6
M11	0.14	6
M3	0.09	5
M2	0.07	0
M10	0.03	4
Average	0.11 ± 0.05	4.0 ± 2.3
No. pregnant		5
% pregnant		83

*Significantly different from low titer values.

Table 5
Pregnancy outcome based on high and low titer serum antibody levels (Experiments 2A,B,C),

Mouse	Serum O.D.value 1/800	Average No. pups/litter
High titer		
M6	0.32	5.0
M4	0.25	1.3
M5	0.21	0.3
M12	0.21	1.0
M7	0.20	0.7
M9	0.19	2.0
Average	0.23 ± 0.05 ($P < 0.002$)*	1.7 ± 1.7 71%↓ ($P < 0.003$)*
Low titer		
M1	0.17	5.7
M8	0.15	8.3
M11	0.14	2.7
M3	0.09	5.7
M2	0.07	7.0
M10	0.03	5.3
Average	0.11 ± 0.05	5.8 ± 1.9

*Significantly different from low titer values.

results of this study indicated that in the 5 mice immunized with P10G (see Materials and methods) there was no detectable antigen specific proliferation of lymph node cells in response to 100 μM of the peptide. The lymph node cells responded to the purified protein derivatives in the complete Freund's

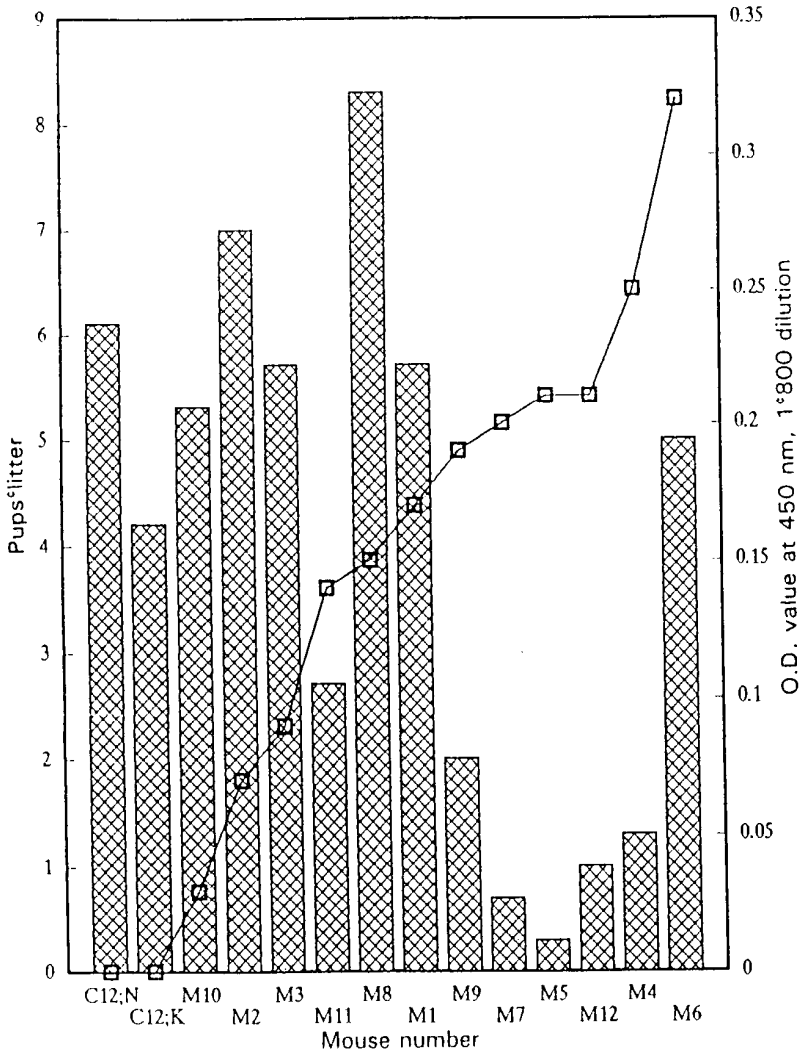


Fig. 1. The relationship between anti-P10G serum values measured at a 1/800-dilution of the serum (O.D., 450 nm) in ELISA and the number of pups/litter can be seen. The number of pups/litter for each P10G-KLH immunized mouse (M1–M12) can be compared to the average number in the KLH-only immunized control mice (C12; K) and the average number in the no injection control mice (C12; N).

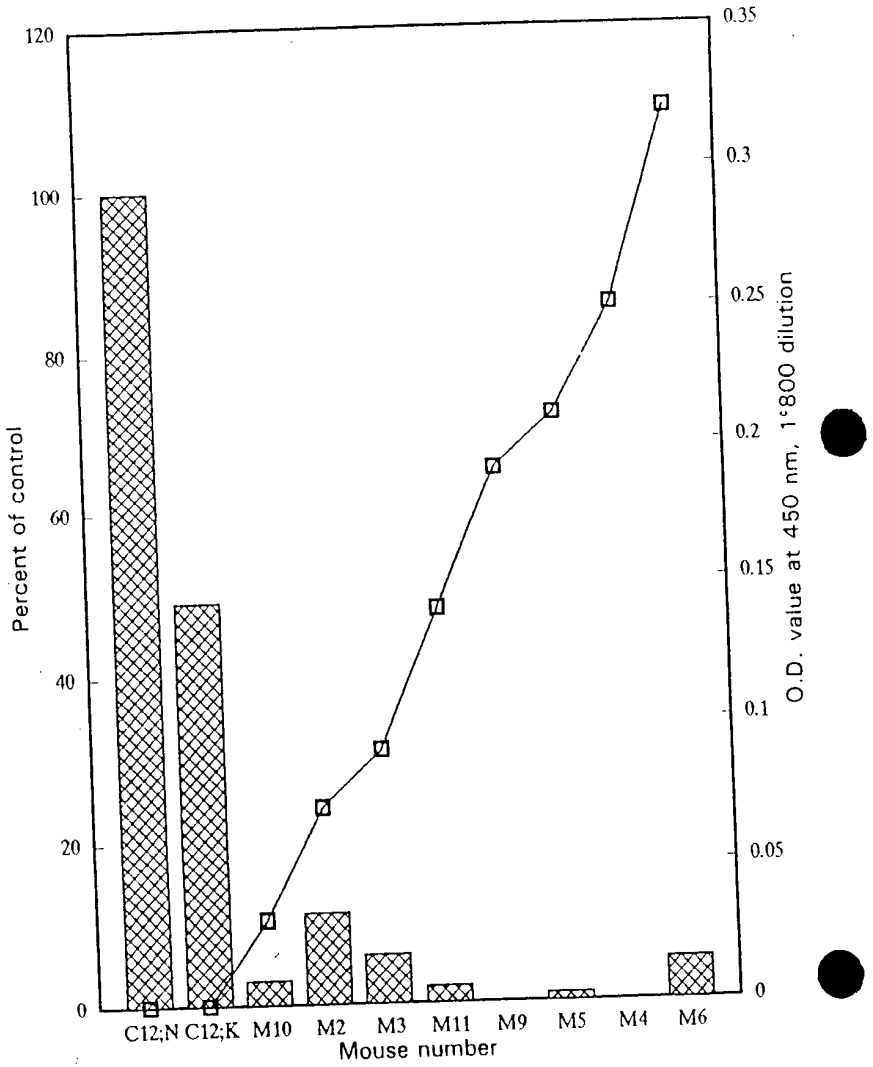


Fig. 2. The relationship between anti-P10G serum values measured at a 1/800-dilution of th serum (O.D., 450 nm) in ELISA and their effect on mouse sperm-zona binding can be seer Serum from P10G-KLH immunized mice (M) can be compared to the pooled serum fro KLH only immunized control mice (C12; K) and the pooled serum from no injection contr mice (C12; N). The binding results were calculated as percent of the no injection control seru pool.

adjuvant. Furthermore, none of these mice developed autoimmune oophoritis. Consequently, it is concluded that P10G is not a T-cell epitope nor does it elicit an autoimmune response in the female mouse.

4. Discussion

This study has demonstrated that female mice can become infertile when immunized with the synthetic peptide P10G conjugated to the carrier protein, KLH. The P10G sequence is derived from a rabbit sperm protein (O'Rand and Widgren, 1990). Importantly, the level of immune response to the peptide is the critical factor in this analysis since without such measurements the overall response of the population to the immunogen would be misleading. In the first experiment (Exp. 1; Table 3) the results were encouraging as it appeared that all mice were going to be infertile. However, the second, larger experiment (Exp. 2; Table 3) demonstrated that this was not the case and that it was important to distinguish those mice with high serum antibody levels from those with lower levels. Two additional immunizations were necessary to distinguish high and low titer subgroups in experiment 2. Infertility was clearly apparent in the high titer subgroup (Tables 4 and 5) both in the last mating and over the course of the entire experiment.

The demonstration that sera from all mice inhibited sperm-zona binding (Fig. 2) and exhibited antisperm antibodies (except M9) reinforces the concept that there is little if any correlation between infertility and the serum titers of antisperm antibodies (Tung et al., 1979) as measured by such in vitro tests. However, this study clearly demonstrates that the degree of immune responsiveness to the well defined B-cell epitope in the immunogen is the critical factor. All the mice in experiment 2 exhibited anti-P10G antibodies (Table 2), yet they had different responses to the presence of spermatozoa in the reproductive tract. It is possible that the high titer subgroup, with more circulating antibodies, had enough antibody present in the reproductive tract to cause infertility. Alternatively, the antibodies may have had some effect on the fertilized egg or its subsequent implantation. Although the parent molecule of P10G, rabbit 14-kDa RSA (O'Rand and Widgren, 1990) has been shown to be specific to testis and spermatozoa (O'Rand et al., 1984), cross-reactivity of anti-P10G antibodies with other tissues within the mouse can not be ruled out. A second alternative explanation for the diversity of responses to P10G and consequently to spermatozoa in the reproductive tract could be a difference in the distribution of B-cells producing antibodies in individual mice. This might have influenced the amount of antibody present and transudation into the female reproductive tract.

Significantly, no T-cell response to P10G was found nor was any ovarian pathology observed. All of the pups born to immunized mice were observed to be normal and healthy. Consequently, P10G elicited a B-cell response that was driven by T-cell epitopes on KLH. This implies that given the proper T-cell epitope to produce high serum titers to P10G, it could be an effective contraceptive without ovarian pathology.

Comparing the present results with previous studies, the average number of pups/litter in the high titer group (1.7 ± 1.7; Table 5) during three matings is comparable to that found by Tsunoda and Chang, (1976; 1.4 ± 4.0) and to that reported by Tung et al., (1979; 2.0) in female mice injected with mouse spermatozoa. In a study of fertility in guinea-pigs immunized with the guinea-pig sperm antigen PH-20, all the immunized animals exhibited serum with extremely high titers to the immunogen as well as inhibition of sperm-zona binding and consequently strong contraceptive effects (Primakoff et al., 1988). More recently, female rats immunized with the human sperm peptide YAL-198 showed a 58% decrease in the pregnancy rate (5/12 pregnant; Vanage et al., 1992). The 17% pregnancy rate (1/6 pregnant) exhibited by the P10G-immunized mice in the last mating of this study is a significant reduction in fertility and warrants further study into the ways in which the number of high responding mice can be increased.

5. Acknowledgments

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Antibody response against epitopes on hCG mapped by monoclonal antibodies in women immunized with an anti-hCG vaccine and its implications for bioneutralization

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Abstract

The immunological determinants on hCG, to which an antibody response is generate women by the contraceptive HSD vaccine, were mapped by using a panel of anti-hCG mo clonal antibodies. Two types of inhibition enzyme immunoassays (EIAs) were used to ana 126 serum samples from 18 subjects immunized with the vaccine and protected from p nancy. Monoclonal antibody (MAb) 357-2, which recognizes β-hCG loop peptide 38-57, not inhibit significantly the binding of immune sera to hCG. Moreover, none of the reacted with this loop peptide in a direct binding EIA, suggesting weak immunogenicit this epitope. All sera competed with MAb 206 and a preponderance of antibodies was against this epitopic region on β-hCG. The antibody titers against the MAb 206 epi showed a good correlation with the bioneutralization capacity of the sera throughout course of immunization. These studies indicate the presence of an immunodominant antig determinant on hCG as recognized by the human immune system.

Key words: hCG vaccine; Human immune response; β-hCG peptide; Monoclonal antib Bioneutralization

1. Introduction

Contraception by evoking a specific immune response against antig crucial for fertility is a feasible proposition. Immunocontraceptive vacci

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Expression and immunogenicity of the V3 loop from the envelope of human immunodeficiency virus type 1 in an attenuated *aroA* strain of *Salmonella typhimurium* upon genetic coupling to two *Escherichia coli* carrier proteins

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A peptide comprising residues glu$_{293}$ to ser$_{334}$ from the principal neutralization determinant (V3 loop) of the envelope of human immunodeficiency virus type 1 (HIV1 LAV$_{BRU}$ isolate) has been inserted within internal permissive sites of either LamB or MalE, two envelope proteins from Escherichia coli *K12. The MalE hybrid protein (MalE133–V3 loop) was stably expresssed in the periplasm of* Escherichia coli *K12, and the V3 loop peptide was detectable on the surface of the native protein by an anti-gp160 monoclonal antibody (mAb 110-A). The disulfide bridge between the two cysteines of the loop was formed. In contrast, genetic coupling to the outer membrane protein LamB did not allow the expression of a stable hybrid protein, and major proteolytic cleavage products of the LamB153–V3 loop were detected by mAb 110-A. The two plasmid-encoded hybrid genes were transferred to an* aroA *mutant of* Salmonella typhimurium. *Constitutive expression of the MalE133–V3 loop had no detectable effect on cell growth and on the survival* in vivo *of the recipient strain. The LamB153-V3 loop was not stably expressed in* Salmonella, *either* in vitro *or* in vivo. *Live recombinant salmonellas expressing MalE–V3 and LamB–V3 loop hybrids were used to immunize mice. The MalE–V3 loop hybrid induced anti-HIV1 envelope antibodies detectable by Western blot and ELISA, while the anti-HIV1 envelope antibodies induced by the LamB–V3 loop hybrid were only detectable by Western blot. In addition, purified MalE–V3 loop hybrid protein was able to stimulate* in vitro *and induce* in vivo *a V3 loop-specific T-cell proliferative response.*

Keywords: HIV1; gp160; *Salmonella* vaccine; LamB; MalE; hybrid protein; epitope

Despite recent advances, the development of a vaccine against human immunodeficiency virus (HIV) remains to be achieved (see Ref. 1 for a review). Most of the targets for neutralizing antibodies were previously shown to lie within the envelope protein gp160. Numerous experiments have now demonstrated that the major neutralization epitope is located in the third variable domain of the gp120 moiety of gp160 (residues 307 to 330) (Refs 2–5 and references therein). This region forms a disulfide-bonded loop, the so-called V3 loop. It contains epitopes recognized by cytotoxic T lymphocytes[6], helper T cells[7,8], and induces antibody-dependent cellular cytotoxicity[9]. This region constitutes a very attractive candidate for the development of a subunit vaccine against HIV.

Synthetic peptides corresponding to this region have been used as immunogens and were shown to allow the induction of high-titre anti-gp160 antibodies, able to neutralize virus infectivity *in vitro*[4,10] and confer protection to chimpanzees *in vivo*[11].

A number of vectors have been developed that allow the expression of foreign polypeptides as genetic fusions to carrier proteins (see Ref. 12 for a review). Fusion of

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peptides from the V3 loop region to viruses or virus-like particles has recently been described[13–15].

We have developed a genetic procedure to express foreign peptides within 'permissive' internal sites of two envelope proteins from *Escherichia coli* K12: the soluble periplasmic MalE protein and the integral outer membrane LamB protein[16]. Humoral and cellular immune responses have been elicited against epitopes inserted in LamB sites exposed at the cell surface or within the MalE protein expressed in the periplasm[17–22]. One of the major advantages of these two carrier proteins is that they can be expressed in enteric bacteria other than *E. coli*, more suitable for vaccine development. In particular, we showed that LamB and MalE hybrids could be expressed in attenuated strains of *Salmonella typhi* and *Salmonella typhimurium*[23]. Attenuated *aroA* mutants of *S. typhimurium*, which induce a broad immune response after oral administration[24], offer a convenient way of presenting heterologous antigens to the immune system. In recent studies, using *Salmonella* as a carrier, several groups have shown that it is also possible to induce an immune response against a number of foreign B-cell or T-cell epitopes, including cholera toxin epitopes[25], malaria antigens[26], hepatitis B surface-antigen epitopes[23,27], tetanus toxin[28], streptococcal M protein[29] and influenza haemagglutinin epitope[30].

We inserted a peptide comprising the V3 loop from the envelope of HIV1 (LAV_{BRU} isolate) at internal sites after residues 153 of LamB and 133 of MalE proteins. The recombinant proteins were expressed in *E. coli* K12 and in the attenuated *S. typhimurium* vaccine strain SL3261[31]. The immunological properties of the V3 loop genetically coupled to these two carrier proteins were studied using live recombinant salmonellas as immunogens. The ability to induce *in vitro* and *in vivo* V3 loop-specific T-cell responses was also tested using purified MalE–V3 loop hybrid protein.

MATERIALS AND METHODS

Bacterial strains and constructions

The LamB and MalE–V3 loop hybrids were first constructed in *E. coli* K12. The recombinant plasmids were then transferred to SL3261 by electroporation[32].

Strain pop 6510 (*thr leu tonB thi lacY1 recA dex5 metA supE*) was used as a recipient for the LamB hybrid constructions. *dex5* is a mutation in gene *lamB* which prevents expression of any detectable LamB protein. Strain PM9F′ (*recA1 endA1 thi gyr196 hsdR17 supE44 relA1* Δ(*lac-proAB*) Δ*malE444 F′ lacI*^{q} *proA*$^+$ *B*$^+$ *lacZ* Δ*M15*) was the recipient for the MalE hybrid constructions. Δ*malE444* is a non-polar deletion in the *malE* gene which prevents expression of any detectable MalE protein.

The double-stranded DNA fragment encoding the V3 loop peptide was obtained by polymerase chain reaction (PCR) amplification on plasmid pRSR3 (kindly provided by Dr S. Wain-Hobson, Institut Pasteur, Paris, France), by a standard procedure[33]. pRSR3 is a derivative of pUC18 carrying a 3845 bp *Eco*RI–*Sac*I fragment comprising the *env* gene from HIV1 (LAV_{BRU} isolate). The two primers used were: 5′ GAA GAT CTT GAA ATT AAT TGT ACA AGA CCC 3′, on the coding strand; and 5′ CGG GAT CCG CTC TAC TAA TGT TAC AAT GTG C 3′, on the non-coding strand. At the 5′ end of the first primer is a *Bgl*II restriction site, and at the 5′ end of the second primer is a *Bam*HI restriction site (in bold). The PCR amplification product was then simultaneously digested with restriction enzymes *Bgl*II and *Bam*HI. The *Bgl*II–*Bam*HI DNA fragment, encoding the V3 loop peptide, was then purified on Tris–acetate–EDTA 1.5% agarose gel, and finally subcloned into the *Bam*HI sites of plasmids pAJC264 (insertions in LamB at site 153[34]), and pTM133 (insertion in MalE at site 133[22]), as described previously[35].

The two hybrid genes are carried on pBR322 derivatives under *ptac* promotor control. In plasmid pAJC264–V3 loop (encoding LamB–V3 hybrid), one *Iq* gene is also present, to maintain a low level of expression in the absence of induction with IPTG. In plasmid pTM133–V3 loop (encoding MalE–V3 hybrid), the *Iq* gene was removed since the construction appeared to be fully stable both *in vitro* and *in vivo* (see below), thus allowing a constitutive expression of the recombinant protein.

The peptide inserted into LamB and MalE will be called the V3 loop, for simplicity, and the two hybrid proteins will be called LamB153–V3 loop, and MalE133–V3 loop (see *Figure 1*).

Intravenous immunizations with live recombinant salmonellas

Balb/c mice were immunized intravenously with approximately 10^7 live recombinant salmonellas (in 0.2 ml PBS). Bacterial were grown in Luria broth[36] containing 100 μg ampicillin ml^{-1}. Expression of the hybrid *lam*B gene was induced by addition of isopropyl-β-D-thiogalactoside (IPTG) (10^{-3} M final) in early exponential growth phase (absorbance at 600 nm, $A_{600} = 0.4$). Growth was pursued until $A_{600} = 1$. Aliquots were snap frozen in liquid nitrogen. Three injections were performed at monthly intervals. Blood samples were collected 7 days after each injection.

The stability *in vitro* of the recombinant plasmids was measured, for the two types of constructions, by plating

Sequence in LamB (insertion between residues 153 and 154 of the mature LamB protein):

```
153                                                    154
TCC CCG GAT CTT ( V3 sequence ) AGA GCG GAT CCC GCC TCT
    P   D   L (E I N C.....C N I S) R   A   D   P   A
```

Sequence in MalE (insertion between residues 133 and 143 of the mature MalE protein):

```
133                                                143
ATC CCG GAT CTT ( V3 sequence ) AGA GCG GAT CCG GGT
    P   D   L (E I N C.....C N I S) R   A   D   P
```

Amino-acid sequences are indicated in the one-letter code.
The dotted line corresponds to the sequence of the V3 loop between the two cysteine residues : TRPNNNTRKSIRIQRGPGRAFVTIGKIGNMRQAH.
The two cysteine residues in the V3 peptide, responsible for the formation of the V3 loop, are in bold letters.
PDL and RADP residues result from the linker sequence.

Figure 1 DNA and amino acid sequences of the LamB–V3 and MalE–V3 loop hybrid proteins in the regions of insertion of the V3 loop

dilutions of fresh bacterial cultures, grown overnight in complete medium with ampicillin, and plated on solid medium with or without ampicillin. Stability *in vivo* was determined in spleens of mice 1 and 14 days after intravenous immunization with 10^5–10^6 colony-forming units (c.f.u.) as previously described[37].

Western blot analysis

Detection of LamB– and MalE–V3 loop hybrid proteins. Immunoblotting of heat-denatured cellular extracts was performed as described previously[34]. For the LamB153–V3 loop hybrid, bacteria were induced by addition of 10^{-3} M IPTG in the early log phase. For the MalE133–V3 loop hybrid, 10^{-3} M IPTG was added only when the recombinant plasmid was expressed in *E. coli* (the recipient *E. coli* strain used contained a *lacIq* gene copy on the F′ episome). The amount of bacterial extract loaded per well corresponded to approximately 5×10^7 bacteria. After electrophoretic transfer to nitrocellulose filters, the extracts were analysed with anti-LamB peptide serum[38] at a dilution of 1/50 000, or anti-MalE serum[39] at a dilution of 1/2000, and with mAb 110-A at a dilution of 1/1000. MAb 110-A (Diagnostic Pasteur, France), was kindly provided by F. Traincard (Institut Pasteur, Paris). MAb 110-A was raised by immunizing mice with recombinant soluble gp160 (Transgène[40]). Its recognition site was mapped within peptide PGRAFVT, at the tip of the V3 loop (Catalogue de Réactifs ANRS, France).

Recognition of recombinant gp160. Sera from mice immunized intravenously with live recombinant salmonellas were tested for their ability to react in Western blot with soluble recombinant gp160. Soluble recombinant gp160 was loaded (200 ng per well), and transferred electrophoretically to nitrocellulose after migration on SDS–polyacrylamide gel. Sera were tested at a final dilution of 1/200. The specificity of recognition of gp160 was also tested in an inhibition assay, by precincubation of serum diluted 1/200 with free V3 synthetic peptide (NTRKSIRIQRGPGRAFVTIGK) at a final dilution of 0.1 mg ml^{-1}, before immunodetection in Western blot. Recognition of gp160–antibody complexes was revealed using anti-mouse IgG coupled to peroxidase (Bio-Rad), as previously described[38].

T-cell proliferation assay

Balb/c mice were immunized subcutaneously at the base of the tail with 10 μg of 18IIIB (315–329) peptide, or 10 μg of hybrid protein MalE133–V3 loop, or 25 μg of wild-type MalE protein emulsified in complete Freund's adjuvant (CFA). Fourteen days later, draining inguinal lymph nodes (LN) were removed aseptically, and 7.5×10^5 LN cells/well were cultured in a 96-well microtitre plate with the appropriate antigen in triplicate in RPMI 1640 medium (Seromed) and 1.5% fetal calf serum (Bayer Diagnostics), 0.5% normal mouse serum, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 2 mM L-glutamine and 5×10^{-5} M 2-mercaptoethanol. After 3 days at 37°C, cells were pulsed for 18 h with 17.5 μCi ml^{-1} of [^{3}H]thymidine and then harvested onto fibreglass filters with an automated cell harvester. Incorporated radioactivity was measured by scintillation counting. Results were expressed as background-subtracted geometric means. Standard deviations of triplicated cultures were <15% of the mean. 18IIIB (315–329) peptide was a kind gift from G. Dadaglio (Institut Pasteur, Paris).

Two to three animals were tested per group, in each experiment. Experiments were repeated at least twice.

ELISA

Recognition of the V3 loop on the surface of native purified MalE133–V3 loop. Ninety-six well microtitration plates (Nunc) were coated overnight with 100 ng of either mAb 110-A or mAb 56.5[22] diluted in PBS. The antibody-coated plates were washed with PBST (PBS with 0.05% Tween 20), blocked for 1 h with PBSTB (PBST with 1% bovine serum albumin), then incubated for 1 h with serial twofold dilutions (0.1–1000 ng/well) of purified wild-type MalE and hybrid MalE133–V3. Wells were washed and incubated for 1 h at 37°C with a 1/1000 dilution (3 μg ml^{-1}) of an immunopurified rabbit polyclonal anti-MalE serum in PBSTB. After washing, wells were incubated with a goat anti-rabbit IgG peroxidase conjugate for 1 h at 37°C. Plates were incubated for 30 min with a substrate solution (TMB kit, KPL). The reaction was stopped by addition of H_2SO_4, and absorbance was recorded at 450 nm.

MAb 56.5 was raised by immunizing mice with purified MalE protein and was kindly provided by F. Traincard (Hybridolab, Institut Pasteur, Paris, France).

Anti-gp160 antibody titres. Each well was coated with 150 ng of soluble recombinant gp160 (Transgène, provided by M.P. Kieny[40]), and serial dilutions of sera from individual mice were tested. Two pools of sera from naive mice, and mice hyperimmunized intravenously with live SL3261 alone, were used as negative controls.

Disulfide bridge formation assay

Reduced cysteines were determined by colorimetric dosage[41]. The MalE133–V3 loop protein was first incubated for 1 h in PBS supplemented with 1% SDS and 10 mM EDTA at room temperature in the presence or absence of reducing reagent dithiothreitol (DTT) (10 mM final). The DTT was removed by gel filtration on a G10 column (Pharmacia). Excess NbS_2 (500 μM) was added to denatured (reduced or not reduced) MalE133–V3 loop (~10 μM). The increase in absorbance at 412 nm following NbS_2 addition was measured against an appropriate blank. Molar absorption coefficients of 14 100 for thionitrobenzoate anion[42] and 64 960 for denatured MalE133–V3 loop hybrid[43] were used.

RESULTS AND DISCUSSION

Properties of the recombinant proteins

Characteristics of the hybrid proteins. Expression of LamB– and MalE–V3 loop hybrid proteins, in *E. coli* K12 and in *S. typhimurium*, was first analysed by Western blotting of heat-denatured bacterial extracts using anti-LamB or anti-MalE rabbit polyclonal sera, and anti-gp160 monoclonal antibody mAb 110-A (see Materials and methods).

With LamB153–V3 loop hybrid, extensive degradation was observed both in *E. coli* and *S. typhimurium*. Different major degradation products (ranging from 20 to 30 kDa) were detected by the anti-LamB serum in the

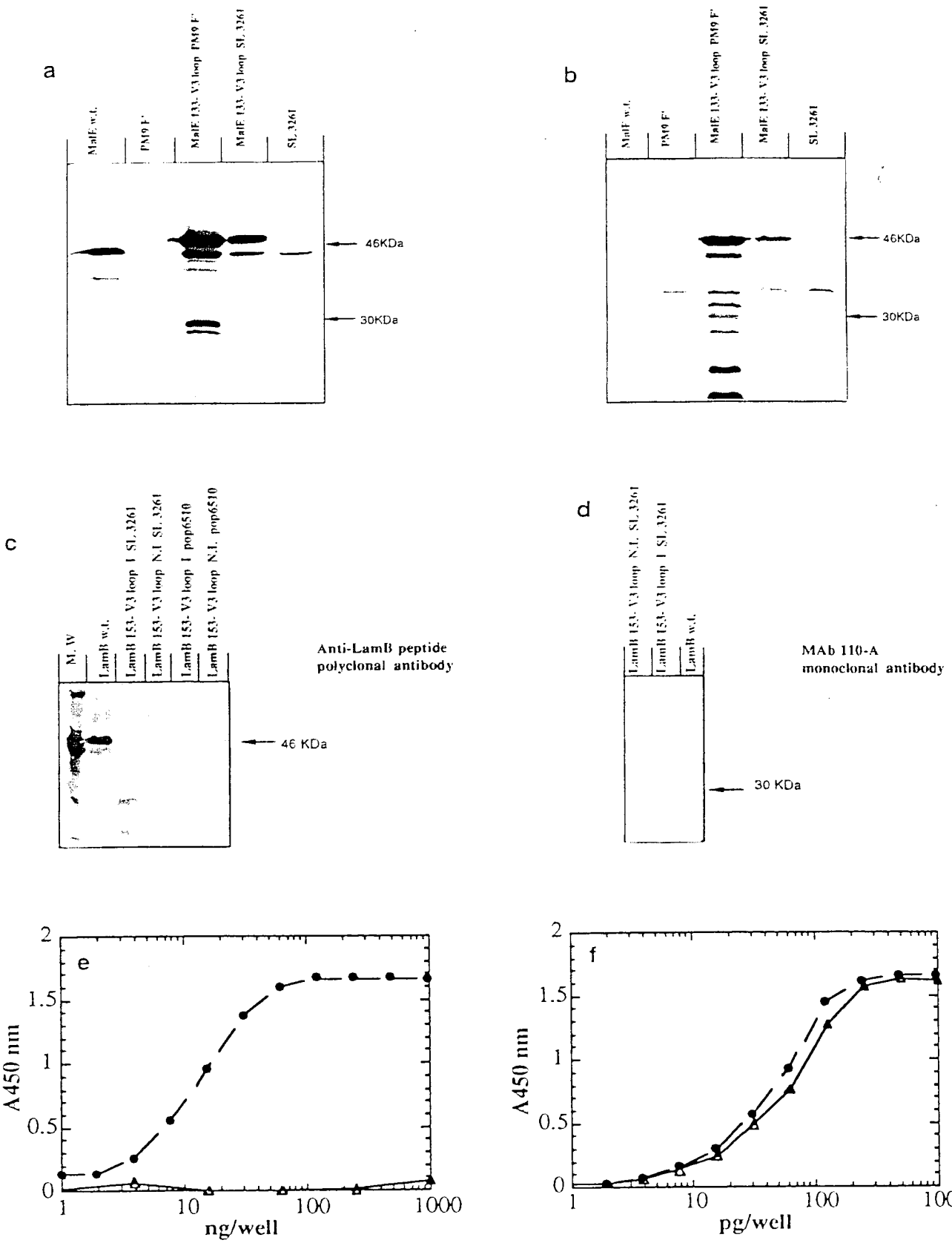

Figure 2 (a)–(d) Western blot analysis of the recombinant proteins. (a)(b) Detection of MalE–V3 loop hybrid with (a) anti-MalE antibody or (b) mAb 110-A. (c)(d) Detection of LamB–V3 loop hybrid with (c) anti-LamB antibody or (d) mAb 110-A. I, induction of LamB153–V3 loop protein expression by addition of IPTG (10^{-3} M final) (see text), NI, no induction. (e)(f) ELISA with native MalE133–V3 loop hybrid protein. Detection of wild-type MalE and MalE133–V3 loop hybrid proteins, in ELISA. (e) MAb 110-A (anti-V3) and (f) mAb 56.5 (anti-MalE) were coated to the ELISA plate. The abcissae indicate the amounts of soluble purified MalE protein or MalE133–V3 protein added per well. The ordinates indicate the absorbance recorded at 450 nm after immunodetection of the bound protein with a rabbit polyclonal anti-MalE antibody followed by a peroxidase-coupled anti-rabbit antibody

two types of extracts. Interestingly, mAb 110-A was able to recognize specifically two degradation bands in *E. coli* (not shown) and in *Salmonella* extracts (*Figure 2d*).

In contrast, MalE133–V3 loop protein was stably expressed, both in *E. coli* and *S. typhimurium* (*Figure 2a–d*). Non-degraded forms of the protein could be detected by anti-MalE and mAb 110-A, in the two strains. However, different major degradation products were also detected by anti-MalE and mAb 110-A, particularly in *E. coli* extracts, probably due to a higher level of expression of the hybrid protein in this strain. MalE133–V3 loop hybrid protein could be purified on an amylose column as described previously for the wild-type protein[44], indicating that the hybrid protein had kept the overall functional and structural properties of wild-type MalE. Some degradation was, however, observed during the course of the purification procedure (approximately half of the hybrid was degraded). The antigenic properties of MalE133–V3 loop protein were then tested by ELISA (see Materials and methods for details). We found that the native hybrid protein was recognized by mAb 110-A (*Figure 2e*), demonstrating that the V3 epitope was expressed at the surface of the protein.

We then tested whether the disulfide bridge between the two cysteine residues flanking the V3 region, naturally formed in the viral envelope protein, was also formed in the MalE hybrid (wild-type MalE does not contain any cysteine residue). For this, the quantity of reduced cysteines per molecule of MalE133–V3 loop hybrid was determined by a colorimetric assay (see Materials and methods). A value of 0.7 reduced cysteines per hybrid protein was found with denatured MalE133–V3 loop protein preparation, and a value of 2.5 with denatured and reduced MalE133–V3 loop protein preparation. This result shows that, in the hybrid protein, more than 70% of the cysteines are engaged in a disulfide bridge. The excess of cysteines found in the denatured and reduced preparation (2.5 cysteines per molecule instead of 2) may be due to an overestimation of the molar absorption coefficient of the protein.

This construction is believed to represent the first example of expression of a foreign disulfide-bridged loop in an internal permissive site of MalE.

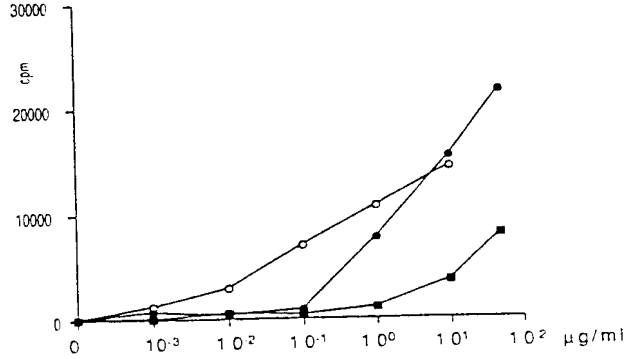

Figure 3 *In vitro* proliferative response of 18IIIB peptide-specific T lymphocytes to the V3 loop region expressed by MalE133–V3 loop hybrid protein. Balb/c mice were immunized subcutaneously with 10 µg of 18IIIB (115–129) synthetic peptide in CFA. Two weeks later, draining LN cells were removed and stimulated *in vitro* with 18IIIB (315–329) peptide (○), MalE133–V3 loop hybrid protein (●) or MalE wild-type protein (■). Proliferation was determined on day 4 by [^{3}H]thymidine incorporation as described

Table 1 *In vivo* stability of the recombinant *Salmonella typhimurium* strains expressing the MalE–V3 and LamB–V3 loop hybrid proteins

	SL3261 (MalE–V3 loop)		SL3261 (LamB–V3 loop)	
Day	– ampi	+ ampi	– ampi	+ ampi
0	5.36	5.31	4.82	4.33
1	3.72 ± 0.21	3.25 ± 0.17	3.78 ± 0.06	3.2 ± 0.13
14	4.3 ± 0.23	4.2 ± 0.28	4.8[a]	3.08 ± 0.42

Viable counts of bacteria present in spleen of mice immunized by the i.v. route with recombinant SL3261 expressing either LamB–V3 loop or MalE–V3 loop proteins were performed at days 1 and 14. Four mice were immunized with each strain. Organ homogenates were prepared and viable counts were made in medium with (+ampi) or without (–ampi) ampicillin to test plasmid segregation *in vivo*. The initial immunizing dose is indicated at day 0. Values are given as $\log_{10}$.
[a]In this case, the standard deviation was not measured because of the high number of viable bacteria in the sample: on each plate, the number of colonies was estimated to be 6000 for 1 ml out of 10 ml spleen suspension. With the LamB-expressing bacteria, the initial dose was lower than with MalE because of cell death upon thawing of the sample

In vitro and *in vivo* stability of the recombinant *Salmonella* strains

While the MalE133–V3 loop construction was stably expressed in *Salmonella in vitro* and *in vivo*, the LamB153–V3 loop hybrid could not be stably expressed (*Table 1*).

In culture, the plasmid carrying the MalE133–V3 loop gene was fully stable and the protein could be expressed constitutively without toxic effect on salmonella growth. In contrast, the recombinant bacteria expressing LamB153–V3 loop hybrid tended to lose the plasmid (1/5 to 1/10 bacteria kept the plasmid after overnight growth, see Materials and methods for details). In spleens of mice collected 14 days after immunization with live recombinant SL3261 expressing MalE133–V3 loop hybrid, all the viable bacteria still carried the plasmid (*Table 1*), while only 1/50 viable bacteria still carried the plasmid after immunization with SL3261 expressing LamB153–V3 loop hybrid (*Table 1*).

In conclusion, MalE appeared to be a more suitable carrier than LamB for the expression of the V3 loop. MalE133–V3 loop protein expressed in SL3261 was not subject to extensive proteolytic degradation, and its constitutive expression had no detectable effect on cell growth and on the *in vivo* survival of the recipient bacteria. In contrast, the LamB153–V3 loop hybrid protein appeared to be highly sensitive to protein degradation, and the recombinant plasmid was unstable *in vivo*. Since it was previously shown that a broad variety of foreign sequences up to about 60 residues in length could be expressed in LamB site 153 without major consequence for the functionality and stability of the protein[45], the instability of this construct might be due to the fact that the loop is formed during exportation of the hybrid polypeptides, preventing its stable incorporation in the outer membrane. The non-correctly exported products would be either rapidly degraded by the bacteria, or the plasmid would be lost, in order to avoid toxicity.

Immunogenicity of the recombinant proteins

Mice were immunized i.v. with live recombinant salmonellas expressing LamB or MalE hybrid proteins.

The humoral responses induced were monitored by Western blot and ELISA. The ability of purified MalE133–V3 loop protein to induce a V3 loop-specific proliferative T-cell response *in vitro* and *in vivo* was also tested.

Antibody responses induced with live recombinant* S. typhimurium *expressing MalE–V3 or LamB–V3 loop hybrids. Intravenous immunization with SL3261 alone did not induce any significant anti-MalE response. Therefore, the anti-MalE antibody responses recorded in sera from the mice immunized with SL3261 expressing MalE133–V3 loop hybrid had been specifically induced by the hybrid MalE protein. In all the sera tested, the anti-MalE antibody titres were very high ($>10^5$, see *Table 2*). The immunized animals also raised anti-gp160 antibodies, as detected by ELISA and Western blotting. In ELISA, four mice out of five raised anti-gp160 antibodies (*Table 2*), with titres ranging from 2×10^3 to 2×10^4 (in the control group of mice immunized with SL3261 alone no significant anti-gp160 antibody response was detected). In Western blot, the sera from the five animals were able to react with gp160, and were inhibited by the synthetic soluble V3 peptide (NTRKSIRIQRGPGRAFVTIGK; *Table 2*).

High anti-LamB antibody responses (of the order of 10^5) were recorded after i.v. immunization of mice with SL3261 expressing LamB153–V3 loop, as well as with SL3261 alone. The high anti-LamB titres also observed in the sera from the control group of mice (immunized with SL3261) were probably due to non-specific crossreaction of anti-*Salmonella* antibodies with the *E. coli* K12 LamB protein preparation (unpublished data). By ELISA, no anti-gp160 antibody response was detected in any of the sera from mice immunized with SL3261 expressing LamB153–V3 loop. However, when two of these sera were tested at a dilution of 1/200 in Western blot against gp160 (see Materials and methods), they were able to react specifically with the recombinant gp160 coated to the nitrocellulose (data not shown).

The increased sensitivity of the Western blot test may be attributed to a more favourable conformation of gp160 coated on the nitrocellulose sheet than adopted on the microtitration plate.

The anti-V3 peptide antibody titres could not be determined, since we found that the pool of sera from mice hyperimmunized with SL3261 alone cross-reacted non-specifically with the synthetic V3 peptide (CTRPNNNTRKSIRIQRGPGRA).

In vivo *and* in vitro *V3 loop-specific T-cell proliferative responses.* The V3 loop contains a dominant T-cell epitope which is located between amino acids 315 and 329 (18IIIB peptide[6]). Therefore, we first examined if 18IIIB peptide-specific T lymphocytes could be stimulated

Table 2 Intravenous immunizations with SL3261 expressing MalE133–V3 loop

Mouse no.[a]	Anti-MalE ELISA titre[b] ($\times 10^5$)	Recognition of gp160 in WB[c]		Anti-gp160 ELISA titre[d]
		–	Inhib. pep.	
1	>3.4	+	+	<1/100
2	>3.4	++	+	$>2 \times 10^3$
3	>4	++	+	1.5×10^3
4	>3.5	+++	+	2×10^4
5	>2.7	+++	+	$>2 \times 10^3$

Titres were recorded in the sera collected after the second booster injection. Values are the reciprocal of the dilution of serum giving a signal twofold that recorded with the pre-immune serum at a dilution of 1/100

[a]Five mice survived after the second booster injection (5/10). The serum of each individual mouse was tested

[b]Anti-MalE antibodies were measured by ELISA with purified wild-type MalE at 100 ng/well, as described previously[19]

[c]Recognition of gp160[4,6] was performed by Western blot at a final dilution of serum of 1/200 (–, left column); +, ++ or +++, recognition of gp160. Inhibition of recognition of gp160 by the serum (Inhib. pep., right column) was performed by preincubating the diluted serum with free V3 synthetic peptide at a final dilution of 0.1 mg ml^{-1}; +, inhibition of recognition of gp160

[d]Recognition of soluble recombinant gp160 by ELISA was performed as described previously[19] by direct coating of gp160 at 150 ng/microtitration

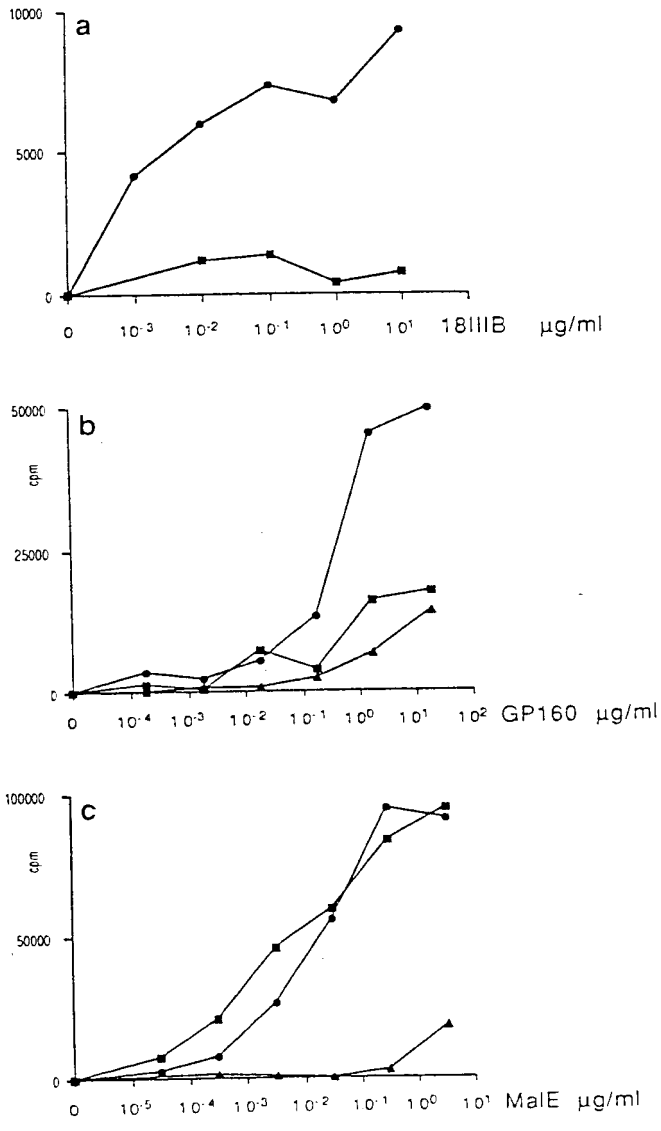

Figure 4 *In vivo* induction of gp160-specific T-cell response by immunization with MalE133–V3 loop hybrid protein. Balb/c mice were immunized subcutaneously with 10 µg of MalE133–V3 loop hybrid protein (●), or wild-type MalE protein (■) in CFA, or CFA alone (▲). Two weeks later, LN cells were removed and stimulated *in vitro* with (a) 18IIIB (115–129) peptide, (b) gp160 protein or (c) wild-type MalE protein. Proliferation was determined on day 4 by [^{3}H]thymidine incorporation

in vitro by recombinant MalE protein expressing the V3 loop. Balb/c mice were immunized with the free 18IIIB synthetic peptide and 2 weeks later LN cells were stimulated *in vitro* with the homologous peptide or with the MalE133–V3 loop hybrid protein. As shown in *Figure 3*, a strong stimulation of proliferative response of LN cells was observed after *in vitro* culture with the recombinant MalE protein. This stimulation was specific to the peptide inserted and was not observed with the wild-type MalE protein.

It was next determined if purified MalE133–V3 loop hybrid protein was able to induce a V3 loop-specific T-cell response *in vivo*. Balb/c mice were immunized subcutaneously with hybrid protein, and LN cells were stimulated *in vitro* with 18IIIB synthetic peptide, recombinant gp160, or wild-type MalE. LN cells from mice primed with MalE133–V3 loop hybrid protein developed a proliferative response *in vitro* against 18IIIB synthetic peptide whereas LN cells from MalE-primed mice did not (*Figure 4a*). This demonstrated that MalE133–V3 loop hybrid could induce *in vivo* and stimulate *in vitro* 18IIIB peptide-specific T-cell proliferative responses.

Moreover, a strong proliferative response was observed when MalE133–V3 loop-primed LN cells were incubated *in vitro* with recombinant gp160. A non-specific response was observed when LN cells from control CFA- or MalE-primed mice were cultured with 2 or 20 $\mu g\ ml^{-1}$ of recombinant gp160. However, this proliferation was considerably lower than that obtained with the LN cells from MalE133–V3 loop-primed mice (*Figure 4b*). A high proliferative response to MalE was obtained with LN cells from both MalE or MalE133–V3 loop-primed mice (*Figure 4c*). These results clearly demonstrated the efficiency of the V3 loop inserted in MalE to induce a specific T-cell response against the envelope protein of HIV1.

Immunosuppression is a major contraindication to the use of live vaccines. It is therefore essential that any live vaccine considered for use against acquired immunodeficiency syndrome (AIDS) be shown to be safe in such immunocompromised hosts. In this respect, attenuated *Salmonella* strains constitute attractive candidates[46]. These strains are safer than vaccinia recombinants[47] and attenuated *Salmonella* have been shown to induce both T-helper and cytotoxic T-cell responses against carried antigens[48].

We have shown that the V3 loop could be stably expressed *in vivo* and *in vitro* in *Salmonella* by genetic coupling to the soluble MalE protein. Intravenous injection of recombinant bacteria expressing the MalE133–V3 loop hybrid induced significantly anti-gp160 antibodies. Moreover, the purified MalE133–V3 loop induced specific proliferative T-cell response against the envelope protein of HIV1. We are at present studying the responses induced against the T-cell epitopes contained in the V3 loop after immunization with live recombinant *Salmonella* expressing the MalE133–V3 loop.

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